

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Clark	Examiner:	McIntosh III
Serial No.:	10/828,753	Group Art Unit:	1623
Filed:	April 21, 2004	Docket No.:	60137.0002USU1
Title:	Modified Fluorinated Nucleoside Analogues		

DECLARATION UNDER 37 CFR 1.132

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Dear Sir:

Now comes Declarant Phillip Furman, Ph.D. who declares and states that:

1. I have 32 years of experience in the discovery, research and development of antiviral/anticancer agents;
2. I received my doctorate in microbiology from Tulane University, and subsequently completed a postdoctoral research fellowship at Duke University;
3. I served as the Director of the Division of Virology at Burroughs Wellcome from 1989 to 1995;
4. I am a named co-inventor of the use of Retrovir® (AZT) for the treatment of HIV and Epivir® (3TC) for the treatment of hepatitis B virus infection;
5. I have been involved in the development of numerous antiviral agents, including Zovirax® (acyclovir), valacyclovir, Retrovir® (AZT), Emtriva™ (FTC), Epivir® for HBV, Ziagen®, and Viroptic®; and
6. I am currently employed as the Vice President of Biological Sciences for Pharmasset, Inc.;

Now comes Declarant Michael J. Sofia, Ph.D. who declares and states that:

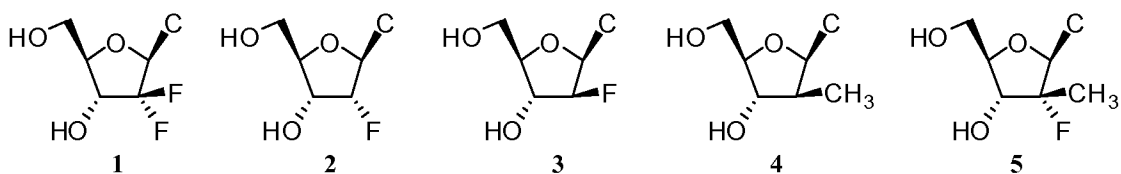
7. I have 21 years of experience in drug research and development across several therapeutic areas including cardiovascular disease, inflammation, and anti-infectives;

8. I received BA degree in 1980 from Cornell University, a Ph.D. in organic chemistry in 1984 from the University of Illinois, Champaign-Urbana, and a NIH Postdoctoral Fellowship at Columbia University;
9. I have been employed in the area of pharmaceutical and/or medicinal chemistry since 1986; and
10. I am currently employed as the Vice President of Chemistry for Pharmasset, Inc.;

Both Declarants also declare and state that:

11. we have read and understood the Office Action issued on March 30, 2007 for 10/828,753 (hereafter "the present application");
12. we have read and understood the disclosure of US 2007/0042939 by LaColla et al. (hereafter "the LaColla published application");
13. we have read and understood the disclosure of the following three provisional applications: (1) 60/392,350, filed June 28, 2002; (2) 60/466,194, filed April 28, 2003; and (3) 60/470,949, filed May 16, 2003;
14. we have supervised and/or directed the preparation of certain compounds and the testing of these compounds as indicated in the Table 2 included below;
15. that we understand that the data presented in the Table 2 included below is intended to support the patentability of the pending claims of the present application.

Compounds considered herein:



Both Declarants further declare and state that:

16. that compound **1** was obtained from HeteroDrugs, Hyderabad, India;
17. the compound **2** was prepared by fluorination of 2,2'-anhydro-ara-cytidine using KF;
18. that compound **3** was obtained from RI Chemicals, Orange, CA, USA;

19. that compound **4** was prepared from 2'-C-methyl cytidine by radical deoxygenation of the 2'-hydroxyl group according to the reference Li, N-S., and Piccirilli, J.A., *J. Org. Chem.*, 2003, **68**, 6799-6802;
20. that compound **5** was prepared as described in the patent applications US 20050009737, WO 2006/012440 and US 2006/0122146;
21. that compounds **1-5** were analyzed for the HCV replicon assay and cell cytotoxicity (Clone A, Hep G2, BxPC3, and CEM), that results of which are presented in the Table 2 included below, using the following protocols;
22. that for HCV Replicon Assay and Clone A cytotoxicity the following materials and reagents were used:

Reagent	Manufacturer	Lot
Assay Medium 90% DMEM 10% FBS 2mM L-glutamine 0.1mM NEAA	HMMS	1145-062
1x PBS w/o Ca and Mg	Invitrogen	1379151
1x Trypsin:EDTA Prepared with PBS	Invitrogen	1289539
DMSO	ATCC	5053545
96-well Tissue Culture Plates	Falcon	3068116
15ml Conical Tubes	Falcon	6181104
50ml Conical Tubes	Falcon	6031485
2ml Collection Tubes	Qiagen	1010310
RNeasy RNA Purification Kit	Qiagen	41831991
b-ME	Calbiochem	B66395
70% EtOH prepared with: 100% EtOH H2O	Acros Pharmasset diH2O	B0512488
Taqman 1-Step RT-PCR Master Mix	ABI	H07783
tRNA Water (60ng/ml) tRNA nuclease free H2O	Roche Fisher	93369502 053607
rRNA Primer Mix (prepared as below)	ABI	0608121
HCV Primer Mix HCV For HCV Rev HCV Probe (prepared as below)	IDT	27478160 27478161 27478170
microamp optical 96well reaction plates	ABI	No lot
Adhesive optical covers	ABI	200509234

23. and that the following procedure was followed:
24. Control Primer and probe mix for rRNA (Applied Biosystems 4308329)
 - a. primer stock (10 μ M) each

- b. probe stock (40 μ M)
 - c. made 29x working solutions in a total volume each of 749.8 μ L (concentration/well is 40 nM)
 - i. 18.75 μ L rRNA probe;
 - ii. 75 μ L primer 1 rRNA
 - iii. 75 μ L primer 2 rRNA
 - iv. 581 μ L of 60 ng/mL tRNA/water
25. Primer probe mix for HCV RNA
- a. probe stock (50 μ M) (6FAM CCTCCA GGA CCC CCC CTC CC TAMRA)
 - b. forward primer stock [100 μ M] (AGC CAT GGC GTT AGT ATG AGT GT)
 - c. reverse primer stock [100 μ M] (TTC CGC AGA CCA CTA TGG)
 - d. made 29x working solution in total volume of 650 μ L: (concentration/well is 400 nM for probe, 1 μ M for each primer)
 - i. 200 μ L probe,
 - ii. 225 μ L forward primer,
 - iii. 225 μ L reverse primer

26. Procedure Day 1

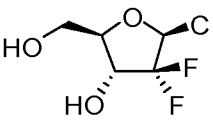
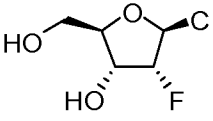
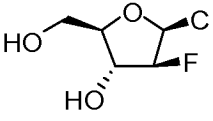
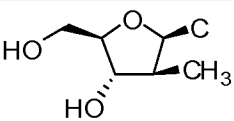
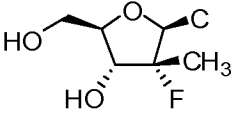
- a. diluted compounds (at 2x) in Assay Medium.
- b. added test and control compounds in 50 μ L into appropriate wells of 96-well plate
- c. harvested cells from T-75 flasks with 1x Trypsin:EDTA.
- d. resuspended in Assay Medium at 1500 live cells/50 μ L/well (30,000 cells/mL)
- e. added 50 μ L of the cell suspension for each well and incubated for 4 days (37°C/5%CO₂/Humidified).

27. Procedure Day 4

- a. removed supernatant and extracted RNA via RNeasy 96 hand book (HMMS1124-008/009),
- b. stored extracted samples at 4°C until RT-PCR (within 24 hours),
- c. amplified RNA for multiplex PCR for HCV RNA in 96 well optical plate,
- d. made master mix/per 96 well plate:
 - i. 1250 μ L Taqman 1 step RT PCR,
 - ii. 68 μ L of prepared HCV primers,
 - iii. 68 μ L prepared rRNA primers,
 - iv. 53 μ L of RNase inhibitor and multiscribe,
 - v. 560 μ L of tRNA/water
- e. added 20 μ L of Master Mix to each well,
- f. added 5 μ L of extracted RNA to appropriate well,
- g. sealed with either flat optical caps or with optical tape
- h. centrifuged briefly to pull mix to bottom of well.
- i. performed RT PCR assay
 - i. 42.0°C for 30mins
 - ii. 95.0°C for 10mins

- iii. 40 Cycles of:
 - 1. 95.0°C for 15secs
 - 2. 60°C for 1min
 - j. determined EC90 and dCt (HCV) and CC50 dCt (rRNA) for each sample.
28. that the 8 day cytotoxicity assay for each of HepG2 (liver), BxPC3 (pancreatic) and CEM (lymphocytic) was performed using the following media and materials as follows:
29. Assay medium for HepG2: DMEM, 10% fetal bovine serum, and 100 IU/mL / 100 µg/mL of Penicillin/streptomycin,
30. Assay medium for BxPC3 and CEM: RPMI-1640, 10% fetal bovine serum, and 100 IU/mL / 100 µg/mL of Penicillin/streptomycin,
31. 96-well cell culture plate
32. CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit
33. ELISA plate reader
34. Methods
- a. added 50 µL of 2X drug dilutions in a 96 well plate (all samples were tested in triplicate)
 - b. Sample dilutions from a 100 mM stock:
 - i. 8 µL of stock + 392 µL of media = 2 mM
 - ii. 200 µL of 2 mM + 200 µL of media = 1 mM
 - iii. 20 µL of 2mM + 180 µL of media = 200 µM
 - iv. 20 µL of 1mM + 180 µL of media = 100 µM
 - v. 20 µL of 200 µM + 180 µL of media = 20 µM
 - vi. 20 µL of 20 µM + 180 µL of media = 2 µM
 - c. In every assay, a "no drug" (medium only) control was used to determine the minimum absorbance values and a "cells + medium only" control was used for the maximum absorbance value. A solvent control was also used in the event the drug was dissolved in DMSO
 - d. cells counted and resuspended in assay medium so that the cells were added at 2000 cells per well,
 - e. added 50 µL of the new cell suspension to each well and incubated the plates at 37°C with 5% CO₂ for 8 days,
 - f. after 8 days of incubation, added 20 µL of MTS dye to each well and incubated the plate for 2 hours at 37°C with 5% CO₂,
 - g. read the plates on an ELISA plate reader at 490 nm.
 - h. calculated the absorbance using the medium only control wells as blanks.
 - i. determined the 50% inhibition value (CC50) by comparing the absorbance in the no-drug cell control wells with the absorbance in wells containing cells and test drug
35. that using the above compounds, materials, and assays that the following data were obtained

Table 2. Activity and Cytotoxicity Comparison of 2'-Substituted Cytidine Analogs

No	Compound	HCV Activity EC ₉₀ (μM)	Cytotoxicity			
			Clone A CC ₅₀ (μM)	Hep G2 CC ₅₀ (μM)	BxPC3 CC ₅₀ (μM)	CEM CC ₅₀ (μM)
1		<1	<0.1	<1	<1	<1
2		5.66	>100	400	10	6
3		Can not determine: Toxic to cells	<50	200	5	5
4		9.73	10.47	40	<1	<1
5		4.5	>100	>1000	>1000	>1000

C represents cytosine.

36. that the data presented in Table 2 for the biological profile of Compound **5**, that includes both the intrinsic potency against HCV and cytotoxicity, can not be predicted based on the structure activity relationship of related compounds;
37. that it is unexpected that appending both a β-methyl group and an α-fluoro substituent to the C-2' position of a 2'-deoxycytidine nucleoside would produce a compound (Compound **5**) that is both a potent inhibitor of HCV replication in cell culture and also lacks cytotoxicity;
38. that compounds containing either a single F atom in either the 2'-β position (Compound **3**) or the 2'-α position (Compound **2**) or containing a di-fluoro

- substitution at C-2' (Compound **1**) demonstrate activity against HCV but also show significant cytotoxicity in one or more cell lines tested;
39. the 2'-deoxycytidine analog (Compound **4**) with a 2'-beta-methyl substituent shows substantial cytotoxicity against all cell line
40. that it is both unexpected and surprising that introducing both a β -methyl and an α -F substituent at C-2' would produce a potent HCV inhibitor with no cytotoxicity when either of these substituents alone produce compounds that show significant cytotoxicity against key cell lines;
41. The undersigned **Declarants** further declare that all statements made herein are of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

Phillip Furman, Ph.D.
Vice President of Biological
Sciences for Pharmasset, Inc.

Michael J. Sofia, Ph.D.
Vice President of Chemistry for
Pharmasset, Inc.

Signature

Signature

Date

Date